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(54) Title: ARGININE-DEPLETED HUMAN TUMOR NECROSIS FACTOR

<u>TNF</u> <u>Mutein</u>	<u>Plasmid</u>	<u>Oligomer</u>
mTNF	pAW711	-----
¶4	pAW736	CACTCGGGTTCGAGACATAAGCTTGCCTGGGCC
¶5	pAW738	GCTTGTCACTCGGGTTCGCATAGCTTGCCTGGGCC
¶6	pAW739	GCTTGTCACTCGGGTCAAGCTTGCCTGGGCC
¶7	pAW737	CAGGCTTGTCACTCGGCATAAGCTTGCCTGGGCC
¶8	pAW740	CTACAGGCTTGTCACTCATAGCTTGCCTGGGCC
¶9	pAW741	GGGCTACAGGCTTGTCCATAAGCTTGCCTGGGCC
¶10	pAW742	CATGGGCTACAGGCTTCAAGCTTGCCTGGGCC
¶11	pAW743	CAACATGGGCTACAGGCTAAGCTTGCCTGGGCC
¶15	pAW744	GAGGGTTTGCTACAACCATAAGCTTGCCTGGGCC
¶156-	pAW745	GATGTTCGTCCTCCTCAGGCAATGATCCCAAAG
¶150-	pAW746	GTATGTTCGTCCTCCTCAGACCTGCCAGACTCGGC
¶140-	pAW747	GTATGTTCGTCCTCCTCAGTCGGCCGATTGATCTC
desSer ₃ desSer ₄	pAW733	GGGTTCGAGAACGGACCATAAGC
Val ₁₅ Ser ₁₆	pAW734	GTTTGCTACAGAACGGCTAC

(57) Abstract

Muteins of tumor necrosis factor (TNF) which are arginine depleted are biologically active and have superior handling properties. Particularly preferred are deletions of, and substitutions by, neutral or acidic amino acids for the arginine residues at positions (2 and 6) of mature TNF. These muteins have higher homogeneity when subjected to isoelectric focusing. Deletion and substitution of arginine at positions (31 and 32) results in TNF muteins which are stable to the action of proteases.

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ARGININE-DEPLETED HUMAN TUMOR NECROSIS FACTOR

This invention relates to the production of tumor necrosis factor muteins. In particular, it concerns production of muteins selectively toxic to tumors, which have improved handling characteristics.

A factor which became familiar as tumor necrosis factor (TNF) was first encountered by Carswell, et al, Proc Natl Acad Sci (USA) (1975) 72:3666. It was found that the sera of endotoxin treated mice, rabbits, or rats which had been previously sensitized with an immunopotentiator such as Bacillus Calmette-Gurin (BCG) contained a substance which, when injected into mice harboring transplanted tumors, caused extensive hemorrhaging of the tumors, without undesirable side effects on the recipient. The sera were thus presumed to contain a substance which was selectively necrotic to tumor cells, and neutral with respect to its reactions with normal tissue, hence the designation, TNF. The ability to cause this selective tumor destruction when injected into whole animals became a standard in vivo assay defining TNF.

TNF has also been produced in cell culture. Matthews, et al, Brit J Cancer (1981) 44:418 were able to obtain TNF activity in the medium of mononuclear phagocytes derived from BCG-injected rabbits; Mannel, et al, Infect Immun (1980) 30:523; ibid (1981) 33:156 obtained TNF activity from the medium of macrophage-enriched peritoneal exudate cells from BCG-infected mice after the cell culture was induced with endotoxin.

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Attempts have been made to purify whatever factor is responsible for the selective cytotoxicity against neoplastic cells but, because the substances are apparently present only in tiny amounts either in the serum of whole animals or in tissue culture media, it has not been possible to effect complete purification. Furthermore, the protein or proteins are evidently unstable, and two recent U.S. patents, 4,447,355 and 4,457,916 are directed to methods for stabilizing the activity of the preparation by addition of, for example, albumin or a carbohydrate material. In the procedures of those disclosures, using standard purification procedures developed by others, it was possible to obtain a specific activity for preparations of TNF to approximately 1×10^6 units/mg, where units were defined in terms of an in vitro assay for cytotoxicity against murine L-M cells (ATCC CCL 1.2). It has not been possible, however, to obtain native material which is both active in the in vivo (Carswell) tumor necrosis assay for TNF and of sufficient purity to permit amino acid sequence information to be obtained.

Indeed, because of the unavailability of pure cytotoxic protein, it is unclear at present how many proteins may be available which are selectively necrotic to cancer cells. The in vivo method of Carswell, et al (supra) has been accepted as the standard defining TNF. Because of the cross-species activity of these factors, this assay is, in one sense, conveniently diagnostic. However, the more conveniently performed in vitro assay for cytotoxicity has frequently been used as an index of TNF activity, despite considerable confusion about whether there exists a one-to-one correlation between the in vitro assay and the in vivo test defining TNF. Indeed, a protein derived from a transformed B-cell line, which is active in the in vitro assay, has been

designated "lymphotoxin", purified to homogeneity and partially sequenced (Genentech, EPO Patent Publication 0100641, published 15 February 1984). It has been assumed that lymphotoxin is a different protein from "TNF" because it is of nonmacrophagic origin. Further, antisera prepared against lymphotoxin do not cross react with the cytotoxic (TNF) factor purified from macrophage (Stone-Wolff, D., et al, J EXP Med (1984) 159:828). This assumption has proved valid, as shown by the lack of sequence homology between lymphotoxin and TNF.

It appears, also, that certain of these factors may exhibit antiparasitic activity; it has been shown that a protein designated TNF, derived from sera of BCG injected mice, exhibits cytotoxic affects on malaria parasites (*Plasmodium falciparum*) in vivo and in vitro (Haidans, et al, Infect Immun (1983) 42:385).

Provision of a defined protein sequence which is capable of a cytotoxic effect specifically directed against tumor cells would, of course, represent a major benefit for both diagnosis and therapy of malignant diseases. To this end, efforts have been made to clone the DNA encoding TNF. A number of groups have apparently succeeded in doing so. PCT application US86/01921 to Cetus, EPO publication 155,549 to Dainippon, EPO application 158,286 to Asahi, EPO application 168,214 to Genentech, and PCT application EPO 85/00721 to Biogen all disclose a substantial portion of human TNF amino acid sequence, as deduced from the cDNA or genomic clone. Provision of this sequence in a form which is stable and easily purified to homogeneity is also desired.

Shirai, T., et al, Nature (1985) 313:803-806 produced a recombinant TNF which, as it turned out, lacks the two N-terminal amino acids of the native protein using an expression vector constructed from DNA

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obtained from a human genomic bank. Other modifications of the amino acid sequence of TNF deduced from the DNA have been suggested in the parent applications hereto, and by others. PCT application Publication No. 5 US86/00236 assigned to Cetus discloses TNF muteins which are cysteine substituted and thus contain alternative amino acids at positions 69 and/or 101. Especially preferred forms disclosed include ser₆₉-TNF, ser₁₀₁-TNF and ser₆₉/ser₁₀₁-TNF. PCT application US85/01921 to 10 Cetus discloses mature TNF and N-terminal-deleted muteins wherein TNF sequences lacking 1-10 amino acids at the N-terminus are active biological forms of TNF. EPO Publication No. 155,549 assigned to Dainippon exemplifies a TNF mutein lacking 2 or 6 amino acids from 15 the N-terminus and discloses -7, -12, -15, and -18 TNF muteins, and EPO Publication No. 158,286 to Asahi also discloses a -2 amino acid TNF. In addition, EPO Publication 168,214 to Genentech mentions a number of 20 TNF muteins, including muteins lacking 2 or 8 amino acids at the N-terminus but shows no data related to their activity. Also suggested (p. 17) is replacement 25 of arginine or lysine at positions 2, 6, 82, 44, 131, 98, 90 or 65 by the basic amino acid histidine in order to prevent proteolysis. This replacement of arg₆ is specifically suggested at p.63, and insertion of Ala between arg₃₁ and arg₃₂ is suggested at p. 64. Finally, 30 PCT application EP85/00721 assigned to Biogen suggests, in general, modifications of the naturally occurring TNF sequence without specifying any particular successful alteration. In all cases, of course, it is recognized that expression in bacteria of the DNA sequence encoding a "mature" form of TNF preceded by a codon for methionine to initiate translation may result in mature proteins which either do not contain the

N-terminal methionine residue, depending on the posttranslational processing in the bacterial host.

As disclosed in Cetus PCT application US86/01921, a human promyelocytic leukemia cell line (HL-60, ATCC No. CCL 240), when appropriately induced, produces a tumor necrosis factor in significant amounts, which has been purified, sequenced, and produced using recombinant techniques. This and other forms of the protein may undergo modest proteolytic cleavage or other modification in the course of purification. It is, according to the present invention, possible to modify the normally encoded TNF sequence to obtain sets of muteins which have significantly improved handling qualities while retaining biological activity.

The invention herein provides a series of muteins which are capable of the range of biological activities exhibited by native TNF, but which exhibit improved stability and ease of purification, thus adding to the convenience of production of clinically useful amounts of pure protein. The muteins of the herein invention are modified from the proteins of native sequence by modification of the arginine residues at positions 2, 6, 31, and/or 32. These modifications, which may be combined with other changes in sequence, such as N-terminal deletions and cysteine substitutions, are helpful in designing a protein having optimum characteristics with respect to preparation of a pure therapeutic material.

Therefore, in one aspect, the invention relates to a human TNF mutein which is modified from the native sequence shown in Figure 1, including the naturally occurring allelic variants thereof, wherein the modifications are selected from:

1) deletion or substitution of the arginine at position 6, optionally in combination with N-terminal

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deletions in positions 1-5, or with cysteine residue substitution;

2) deletion or substitution of the arginine residue at position 2, optionally in combination with deletion of the N-terminal amino acid and/or cysteine substitution;

3) deletion or substitution of the arginine residues at both positions 2 and 6 with corresponding optional additional modifications; and

4) deletion or substitution of the arginine at positions 31 or 32 or both, optionally in combination with the foregoing deletion or substitutions at positions 2 and/or 6, and optionally in combination with additional N-terminal deletions or cysteine substitution;

wherein the amino acid substituted for arginine is a neutral or acidic amino acid.

In addition to or in lieu of the foregoing modifications, the valine at position 1 of the TNF shown in Figure 1 may be replaced by an alternative neutral amino acid, such as leucine, or an acidic amino acid, such as glutamic.

In other aspects, it relates to homogeneous preparations of DNA sequences encoding these proteins, to recombinant DNAs capable of effecting their expression, to transformation vectors capable of conferring on a transformant host the ability to express these TNF muteins, the recombinant hosts so transformed, and to methods of obtaining the various compositions of the invention.

One particular allelic variation of the particular human TNF depicted in Figure 1 has the N-terminal sequence Val-Arg-Ser-Arg-Thr-Pro-Ser-Asp-Lys-Pro-Val-Ala-Val-Ser-Val-Ala-Asn-Pro-; the corresponding arginine depleted muteins (especially arginines shown here

at positions 2 and 4) of this native sequence are also included in the invention.

The invention also relates to pharmaceutical compositions containing TNF and to methods of treatment using these compositions.

Figure 1 shows the complete nucleotide sequence of pE4 and the amino acid sequence for human TNF deduced from it.

Figure 2 tabulates plasmids for expression of DNA starting materials for the arginine-depleted muteins of the invention and oligomers used to construct them by site specific mutagenesis.

Figure 3 tabulates expression plasmids for the arginine-depleted muteins of the invention and the corresponding primer nucleotides.

Figure 4 shows SDS gels performed on purified recombinant TNF (rTNF) muteins.

Figures 5 and 6 show the results of isoelectric focusing (IEF) gels performed on purified rTNF muteins.

A. Definitions

As used herein, "tumor necrosis factor" (TNF) refers to an amino acid sequence equivalent to or a mutein of that shown in Figure 1 and the naturally occurring allelic variants thereof, which is capable of selective cytotoxicity against tumor cells. TNF must be active in the in vitro cytotoxicity assay based on the continuous murine connective tissue cell line L-929 as described hereinbelow. It is recognized that this definition of TNF activity is not precisely the same as that set forth in the disclosure coining this term by Carswell, et al (supra). However, this activity as confirmed by the in vitro cytotoxicity assay against human tumor cells provides sufficient assurance of

5 utility that qualification as a tumor necrosis factor using this assay is justified. As set forth hereinbelow, the cytotoxicity against L-929 appears to generalize to other human tumors. It is expected that there is a substantial overlap between factors active in the specified cytotoxicity assay and the in vivo assay outlined by Carswell. The TNF active in this assay can be distinguished from, for example, lymphotoxin, by immunospecificity or amino acid sequence homology, as 10 set forth herein.

15 The TNF protein of the invention, depending on the pH of its environment, if suspended or in solution, or of its environment when crystallized or precipitated, if in solid form, may be in the form of pharmaceutically acceptable salts or may be in neutral form. The free amino groups of the protein are, of course, capable of forming acid addition salts with, for example, inorganic acids such as hydrochloric, phosphoric, or sulfuric acid; or with organic acids such as, for example, acetic, glycolic, succinic, or mandelic acid. The free 20 carboxyl groups are capable of forming salts with bases, including inorganic bases such as sodium, potassium, or calcium hydroxides, and such organic bases as piperidine, glucosamine, trimethylamine, choline, and 25 caffeine. In addition, the protein may be modified by combination with other biological materials such as lipids and saccharides, or by side chain modification such as acetylation of amino groups, phosphorylation of hydroxyl side chains, hydroxylation of proline residues, or oxidation of sulfhydryl groups. All of these 30 modifications are included within the scope of the definition, so long as the TNF activity is retained.

35 It is, of course, understood that minor modifications of primary amino acid sequence may result in proteins which have substantially equivalent or

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enhanced activity as compared to the sequence set forth in Figure 1. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental such as through mutation in hosts which are TNF producers. All of these modifications are included as TNF muteins, as long as TNF activity, as above defined, is retained and as long as homology with the sequence of Figure 1 exceeds 80%. This is an arbitrary boundary, but a reasonable approximation.

10 TNF Muteins

Although it is also somewhat arbitrary, the 157 amino acid sequence for human TNF shown in Figure 1 can be used as a starting point for discussion and will be designated herein "mature" or "mTNF".

15 It has been shown that muteins lacking up to the first ten amino acids at the N-terminus of the sequence shown in Figure 1 (Val-Arg-Ser-Ser-Ser-Arg-Thr-Pro-Ser-Asp) have comparable or greater specific activities as compared to the TNF of the sequence shown. The pattern of specific activities appear to follow a bell-shaped curve with an optimum activity when 6-8 N-terminal amino acids are deleted depending on the assays and conditions used. In addition, deletions from the C-terminus of TNF as shown in Figure 1 may be 20 harmless. Cysteine-substituted muteins of the "mTNF" of Figure 1 are also biologically active. In general, neutral amino acid replacements of the cysteine at position 69 result in active TNF proteins. It appears that the cysteine at position 101 is also dispensable, and muteins having alternative neutral amino acids in 25 this positions, as well as muteins wherein both cysteines 69 and 101 have been replaced or deleted have 30 been prepared.

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5 All of these muteins can be modified according to the method of the invention herein to obtain arginine-depleted forms which retain TNF activity and may have enhanced specific activity in vitro and in vivo; instead or in addition, the valine at position 1 can be replaced by a different neutral amino acid or an acidic amino acid.

10 Finally, genes have been constructed wherein the TNF purified from native sources is used as a model. Thus, genes encoding a mutein wherein one or two serine residues at positions 3 and 4 are deleted have been prepared and the corresponding arginine depleted muteins of these forms are within the scope of the invention.

15 Notation

For convenience, as stated above, the protein having the amino acid sequence number 1-157 in Figure 1 will be used as a reference and designated, perhaps arbitrarily, mTNF (mature TNF). All other amino acid sequences having the requisite homology with mTNF, and showing TNF biological activity, will be referred to as "muteins" of mTNF and will be denoted as to their differences from mTNF using the numbering of residues shown in the figure.

20 For example, muteins which have substitutions for cysteine at position 69 will be denoted using the substituted residue and the position number, e.g., peptides having a serine in place of the cysteine at position 69 are designated ser₆₉-TNF. If a residue is simply missing, it will be renamed des-residue, so that, 25 for example, the mutein wherein the serines at positions 3 and 4 are deleted will be designated des-ser₃des-ser₄-TNF. Muteins which lack segments of amino acids at the N- or C-terminus are denoted according to the terminus affected. Deletions at the N-terminus will be shown as-

lacking the appropriate number of amino acids using "NV" followed by the number missing. For example, muteins which lack one N-terminal amino acid as compared to the protein shown in Figure 1 will be designed NV 1TNF. For 5 deletions at the C-terminus, a "CV" will be followed by the number of the last remaining residue and a minus sign. Thus for the mutein having 7 amino acids removed from the C-terminus, the designation would be CV150⁻-TNF. Where combinations of the foregoing 10 alterations are made, the designation shows all of them, e.g., NV₁des-ser₃des-ser₄ser₆9CV150⁻-TNF.

Not all muteins of mTNF are recombinantly or deliberately produced. Indeed, as will be noted by comparing the sequence obtained for the N-terminal amino acids of the HL-60 secreted TNF with the corresponding portion of the deduced sequence set forth in Figure 1, minor modifications appear in the primary structure although both proteins exhibit TNF activity.

Specifically, the deduced sequence has an additional 20 pair of serine residues following the serine at position 3 before resuming the homology shown between positions 4-12 of the HL-60 derived protein and positions 6-14 of the deduced sequence. In addition, positions 13 and 14 of the HL-60 derived protein are val-ser; the corresponding positions 15 and 16 of the deduced 25 sequence are his-val. To conform the designation of mTNF, this sequence could be called des-ser₃-des-ser₄-val₁₅-ser₁₆- TNF.

"Operably linked" refers to a juxtaposition wherein the components are configured so as to perform their usual function. Thus, control sequences operably linked to coding sequences are capable of effecting the expression of the coding sequence.

"Control sequence" refers to a DNA sequence or 35 sequences which are capable, when properly ligated to a

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desired coding sequence, of effecting its expression in hosts compatible with such sequences. Such control sequences include promoters in both procaryotic and eucaryotic hosts, and in procaryotic organisms also include ribosome binding site sequences, and, in eucaryotes, termination signals. Additional factors necessary or helpful in effecting expression may subsequently be identified. As used herein, "control sequences" simply refers to whatever DNA sequence may be required to effect expression in the particular host used.

"Cells" or "recombinant host" or "host cells" are often used interchangeably as will be clear from the context. These terms include the immediate subject cell, and, of course, the progeny thereof. It is understood that not all progeny are exactly identical to the parental cell, due to chance mutations or differences in environment. However, such altered progeny are included when the above terms are used.

"Acidic or neutral" amino acids, which substitute for the designated arginine residues herein, or for the N-terminal valine, refers to those which are neutral or negatively charged at pH 7. Generally this category includes the 17 of the 20 encoded amino acids other than arg, his and lys, as well as their derivatives, such as hydroxyproline, which have the required ionization status. Preferred for the substitution at positions 2, 6, 31, and 32 are glu, gln, asp, asn, ser, pro, and gly. Particularly preferred at positions 2 and 6 are asp and glu; at positions 31 and 32, gln and asn, and at position 1, leu and ile.

B. Starting Materials and Preferred Embodiments

The starting materials containing DNA sequences encoding the recombinant mTNF and certain

muteins thereof have been described and are disclosed in PCT application Publication No. US86/01921 assigned to the assignee herein. This publication also gives details as to procedures for obtaining mutated forms of the mTNF and its disclosure is incorporated herein by reference. Also disclosed in the cited application is a summary of general methods of manipulating recombinant DNA, and of various hosts and expression vectors usable in a variety of systems to obtain the proteins. These directions are incorporated herein by reference as well; they are, in addition, generally known in the art.

In general, the modified forms or muteins of TNF of the present invention can be prepared in a variety of hosts, including bacteria, yeast, mammalian cells, and other eucaryotic systems. Suitable control sequences appropriate to each of these systems is known in the art, and any convenient set of control sequences may be ligated to the DNA sequences of the invention herein which encode the desired muteins in order to effect their expression.

The TNF muteins of the invention can be formed as mature intracellular proteins by preceding their coding sequences with a methionine codon. The resulting methionine residue may or may not be removed during processing; accordingly, the protein forms claimed are understood to be claimed as the specified sequences with or without N-terminal methionine included. Also included are preparations in which some molecules retain the methionine while others do not.

The TNF may also be produced in secreted form, in which event an appropriate signal sequence is encoded into the DNA sequence upstream of the desired mutein. Signal sequences appropriate to the various hosts which may be used for expression are known in the art, such as the penicillinase or phosphatase A sequences in

bacteria, hormone signal sequences such as the HGH signal sequence in mammalian cells, alpha-factor signal sequence in yeast, and so forth.

5 Although not favored, the TNF mutoins of the invention may also be prepared as fusion proteins, wherein the claimed sequences are understood to be extended at the N or C terminus or both by additional peptide not related to TNF.

10 The DNAs encoding the mutoins of the invention are obtained from the DNA encoding the disclosed forms of TNF using site-directed mutagenesis techniques as described in detail below. Additional modifications can also be made using these same techniques, which are well established and widely practiced in the art.

15 C. TNF Mutoins of the Invention

20 Preferred forms of the mutoins of the invention include those wherein the arginine at position 2, position 6, or both (referencing the numbering system of Figure 1) is either deleted or substituted by a neutral or positively charged amino acid. Neutral or positively charged amino acids are those so described in their status at pH 7. Generally this category includes the 17 of the 20 encoded amino acids other than arg, his and lys, as well as their derivatives, such as hydroxyproline, which have the required ionization status.

25 Also preferred are forms of the invention wherein the mTNF of Figure 1 is modified by substitution or deletion of the arginine residues at positions 31 and/or 32 and wherein, again, any substitution is by neutral or acidic amino acid residues rather than a basic amino acid residue such as lysine.

30 In addition, the valine at position 1 of mTNF may be replaced by another neutral amino acid, such as

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leucine or isoleucine, preferably leucine, or by an acidic amino acid.

Also preferred are combinations of the foregoing modifications with N-terminal deletions appropriate to the mutein involved. Particularly preferred muteins of the invention include:

5 glu₆-TNF
10 pro₆-TNF
 asp₆-TNF
 ser₆-TNF
 gly₆-TNF
 N₇5 glu₆-TNF
 N₇5 pro₆-TNF
 N₇5 asp₆-TNF
15 N₇5 ser₆-TNF
 N₇5 gly₆-TNF
 N₇4 glu₆-TNF
 N₇4 pro₆-TNF
 N₇4 asp₆-TNF
20 N₇4 ser₆-TNF
 N₇4 gly₆-TNF
 N₇3 glu₆-TNF
 N₇3 pro₆-TNF
 N₇3 asp₆-TNF
25 N₇3 ser₆-TNF
 N₇3 gly₆-TNF
 N₇2 glu₆-TNF
 N₇2 pro₆-TNF
 N₇2 asp₆-TNF
30 N₇2 ser₆-TNF
 N₇2 gly₆-TNF
 leu₁glu₆-TNF
 leu₁pro₆-TNF
 leu₁asp₆-TNF
35 leu₁ser₆-TNF

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leu1gly6-TNF
glu6ser69-TNF
glu6ser101-TNF
glu6ser69ser101-TNF
5 pro6ser69-TNF
pro6ser101-TNF
pro6ser69ser101-TNF
asp6ser69-TNF
asp6ser101-TNF
10 asp6ser69ser101-TNF
ser6ser69-TNF
ser6ser101-TNF
ser6ser69ser101-TNF
gly6ser69-TNF
15 gly6ser101-TNF
gly6ser69ser101-TNF
N⁴ glu6ser69-TNF
N⁴ glu6ser101-TNF
N⁴ glu6ser69ser101-TNF
20 N⁴ pro6ser69-TNF
N⁴ pro6ser101-TNF
N⁴ pro6ser69ser101-TNF
N⁴ asp6ser69-TNF
N⁴ asp6ser101-TNF
25 N⁴ asp6ser69ser101-TNF
N⁴ ser6ser69-TNF
N⁴ ser6ser101-TNF
N⁴ ser6ser69ser101-TNF
N⁴ gly6ser69-TNF
30 N⁴ gly6ser101-TNF
N⁴ gly6ser69ser101-TNF
glu2-TNF
leu1glu2-TNF
leu1-TNF
35 ser2ser6-TNF

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ser₂ser₆ser₆₉-TNF
ser₂ser₆ser₁₀₁-TNF
ser₂ser₆ser₆₉ser₁₀₁-TNF
gly₂gly₆-TNF
5 gly₂gly₆ser₆₉-TNF
gly₂gly₆ser₁₀₁-TNF
gly₂gly₆ser₆₉ser₁₀₁-TNF
glu₂glu₆-TNF
glu₂glu₆ser₆₉-TNF
10 glu₂glu₆ser₁₀₁-TNF
glu₂glu₆ser₆₉ser₁₀₁-TNF
asp₂asp₆-TNF
asp₂asp₆ser₆₉-TNF
asp₂asp₆ser₁₀₁-TNF
15 asp₂asp₆ser₆₉ser₁₀₁-TNF
glu₃₁-TNF
glu₃₂-TNF
glu₃₁glu₃₂-TNF
gln₃₁-TNF
20 gln₃₂-TNF
gln₃₁gln₃₂-TNF
ser₃₁-TNF
ser₃₂-TNF
ser₃₁ser₃₂-TNF
25 N₇4 glu₃₁-TNF
N₇4 glu₃₂-TNF
N₇4 glu₃₁glu₃₂-TNF
N₇4 gln₃₁-TNF
N₇4 gln₃₂-TNF
30 N₇4 gln₃₁gln₃₂-TNF
N₇4 ser₃₁-TNF
N₇4 ser₃₂-TNF
N₇4 ser₃₁ser₃₂-TNF

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and the foregoing position 31 and/or 32 arginine-depleted muteins wherein the above-listed N-terminal modifications are also present.

Also preferred are

5 glu₂des-ser₃des-ser₄-TNF;
 leu₁glu₂des-ser₃des-ser₄-TNF;
 des-ser₃des-ser₄glu₆-TNF;
 des-ser₃des-ser₄pro₆-TNF;
 des-ser₃des-ser₄asp₆-TNF;
10 des-ser₃des-ser₄ser₆-TNF;
 des-ser₃des-ser₄gly₆-TNF;
 pro₂des-ser₃des-ser₄-TNF;
 leu₁des-ser₃des-ser₄-TNF;

D. Assays

15 Cytotoxic Assay Procedure

The L-929 assay system is an improved convenient in vitro assay which permits rapid measurement of TNF activity. Its degree of correlation with the in vivo tumor necrosis assay of Carswell is, at present, unknown; however, as it utilizes murine tumor cells specifically, the correlation is expected to be high. The protein designated lymphotoxin in EPO publication no. 0100641 (supra) also gives activity in this assay. The assay is similar in concept to that disclosed in U.S. 4,457,916 which used murine L-M cells and methylene blue staining. However, the L-929 assay has been shown to correlate (for HL-60-derived TNF) with human tumor cell line cytotoxicity.

30 In the L-929 assay system herein, L-929 cells are prepared overnight as monolayers in microtiter plates. The test samples are diluted 2-fold across the plate, UV irradiated, and then added onto the prepared cell monolayers. The culture media in the wells are then brought to 1 µg/ml actinomycin D. The plates are

allowed to incubate 18 hr at 37°C and the plates are scored visually under the microscope. Each well is given a 25, 50, 75 or 100% mark signifying the extent of cell death in the well. One unit of TNF activity is defined 5 as the reciprocal of the dilution at which 50% killing occurs.

In addition, a more sensitive version of this assay monitors the release of ³⁵S labeled peptides from prelabeled cells, when treated with the test sample and actinomycin D. This version of the assay can be used to 10 quantitate potency, e.g., to evaluate the relative potency of oocyte translated material. Briefly, actively growing L-929 cultures are labeled with ³⁵S 15 methionine (200 μ Ci/ml) for 3 hr in methionine-free media supplemented with 2% dialyzed fetal calf serum. The cells are then washed and plated into 96 20 plates, incubated overnight, and treated the with 2-fold dilutions of test samples and 1 μ g/ml actinomycin D. The cultures are then incubated at 37°C 25 for 18 hr. 100 μ l supernatant aliquots from each well are then transferred onto another 96 well plate, acid (TCA) precipitated, and harvested onto glass fiber filters. The filters are washed with 95% ethanol, dried and counted. An NP40 detergent control is included in every assay to measure maximum release of radioactivity 30 from the cells. The percent ³⁵S release is then calculated by the ratio of the difference in count between the treated cells and untreated controls divided by the difference between NP40 treated cells and untreated controls, i.e., by the ratio:

$$\% \text{ release} = \frac{\text{sample cell control}}{\text{NP40 cell control}} \times 100.$$

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Higher TNF potency results in higher values of this ratio.

The foregoing assay is conveniently modified to use human tumor cell lines as subject cells. Units are defined and % release calculated in the same way as for L-929 cells above.

In vivo assays

Preparations may also be tested for TNF activity using the ability of this substance to kill or repress the growth of tumors and to protect the animal bearing the tumor from mortality. Balb/c mice are injected subcutaneously with various types of tumor cells to create a localized tumor. Tumor cell lines included MethA mouse fibrosarcoma, obtained as a cell suspension from ascites fluid, and MCF-7 a human breast carcinoma which is administered as a 1 mm³ clump of cells.

For the assay, female Balb/c mice (19-22g) are injected subcutaneously by 26 gauge needle with either suspension containing 5x10⁵ fibrosarcoma cells in 0.1 ml medium or with the MCF-7 clumps (the fibrosarcoma suspension is prepared from 8 day old ascites fluid by cell counting and dilution with serum free medium). After 9-10 days, when the tumor became palpable, amounts of TNF to be tested (in the range of 1 µg per mouse) are injected IV, and administration of TNF repeated, if desired, on subsequent days. Results are assessed by measuring tumor volume and by survival rate.

E. Utility and Administration of the Muteins of the Invention

The TNF muteins of the invention have similar biological activity to the mTNF and native TNF proteins

and are thus useful therapeutically in purified forms in the treatment of tumors.

The TNF proteins may be purified by conventional methods or by the protocols described in EPO Publication No. 220,966 published 6 May 1987, describing (1) purification by passing TNF-containing fluid through a continuous hydrophobic porous matrix and recovering the TNF, wherein TNF comprises in the range of 40-50% of the total protein and the TNF has an endotoxin content in the range of 10 ng/ml to 10 μ g/ml, and (2) a purification scheme utilizing ion exchange chromatography, HPLC and gel filtration, as well as analysis by isoelectric focusing. With regard to such purification, the TNF muteins of the invention have superior properties to mTNF in that they are more easily produced as "clean" products. Whereas purified recombinant mTNF shows a family of proteins with apparent side chain modifications, as evidenced by behavior on isoelectric focusing (IEF) gels, these muteins give essentially single bands when subjected to this procedure. Also, proteolytic cleavage is prevented.

As is the case for mTNF, the TNF muteins of the invention can be formulated and administered in conventional ways known in the art. Suitable formulations may be found, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, latest edition. Administration is typically intravenous or otherwise parenteral (e.g., subcutaneous, intravascular), but local administration to the tumor is also feasible in some cases.

In one formulation, the TNF may be reacted with a homopolymer or copolymer of polyethylene glycol or a polyoxyethylated polyol, provided that the polymer is soluble in water at room temperature. The polymer is

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reacted first with a coupling agent having terminal groups reactive with both the free amino or thiol groups of the protein and the hydroxyl group of the polymer. Examples of such coupling agents include hydroxynitrobenzene sulfonic ester, cyanuric acid chloride, and N-hydroxysuccinimide. The TNF is then formulated directly with the water-soluble carrier and buffer as described above, and the formulation may be lyophilized and the lyophilized mixture reconstituted as described above.

The dosage for the TNF will depend on many factors--for example, on the type of host and type of cancer, route, schedule and sequence of administration, existing tumor burden, the type of TNF, and the level of toxicity tolerated. Toxicity to the host may be defined, for example, by the extent and type of side effects or by the amount of body weight loss. If body weight loss is the criterion for toxicity, typically a loss of from 10-15% by weight will be tolerated, with greater than 15% loss being considered toxic. For an illustrative set of parameters, if body weight loss of greater than 15% is considered toxic, if the host is murine, if the route of administration is intravenous via a sequential administration every third day for three times, the dosage level at each administration of recombinant, microbially produced TNF is about 25-100 μ g/kg host TNF.

For parenteral administration the TNF will generally be formulated in a unit dosage injectable form (solution, suspension, emulsion), preferably in a pharmaceutically acceptable carrier medium which is inherently nontoxic and nontherapeutic. Examples of such vehicles include water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate

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5 may also be used. The carrier medium may contain minor amounts of additives such as substances which enhance isotonicity and chemical stability, e.g., buffers and preservatives. The TNF will typically be formulated in such carriers at a concentration of about 0.5 mg/ml to 20 mg/ml.

F. Illustrative Muteins

10 Particular representative muteins are prepared below in recombinant hosts. Host strains used in cloning and expression herein are as follows:

15 For cloning and sequencing, and for expression of construction under control of most bacterial promoters, E. coli strain MM294 (supra), Talmadge, K., et al, Gene (1980) 12:235; Meselson, M., et al, Nature (1968) 217:1110, was used as the host. For expression under control of the PLNRBS promoter, E. coli strain K12 MC1000 lambda lysogen, N7N53cI857SusP80, ATCC 39531 (hereinafter sometimes referred to as MC1000-39531) is used.

20 For M13 phage recombinants, E. coli strains susceptible to phage infection, such as E. coli K12 strain DG98, are employed. The DG98 strain has been deposited with the American Type Culture Collection, Rockville, MD (ATCC) July 13, 1984 and has accession number 39,768.

25 The DNA encoding the representative muteins of the invention was constructed using site-specific primer-directed mutagenesis. DNAs encoding additional muteins to those illustrated below are constructed in a similar manner. This is conducted using a primer synthetic oligonucleotide complementary to a single-stranded phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. The synthetic oligonucleotide is used as a primer to direct

5 synthesis of a strand complementary to the phage, and the resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells which harbor the phage.

10 Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form; 50% will have the original sequence. The resulting plaques are hybridized with kinased synthetic primer at a temperature which permits hybridization of an exact match, but at which the mismatches with the original strand are sufficient to prevent hybridization. Plaques which hybridize with the probe are then picked, 15 cultured, and the DNA recovered. Details of site-specific mutation procedures are described below in specific examples.

20 The mutagenized DNAs are then ligated into expression vectors. In the constructions set forth below, correct ligations for plasmid construction are confirmed by first transforming E. coli strain MM294 obtained from E. coli Genetic Stock Center, CGSC #6135, or other suitable host with the ligation mixture. 25 Successful transformants are selected by ampicillin, tetracycline or other antibiotic resistance or using other markers depending on the mode of plasmid construction, as is understood in the art. Plasmids from the transformants are then prepared according to the method of Clewell, D. B., et al, Proc Natl Acad Sci (USA) (1969) 62:1159, optionally following 30 chloramphenicol amplification (Clewell, D. B., J Bacteriol (1972) 110:667). The isolated DNA is analyzed by restriction and/or sequenced by the dideoxy method of 35 Sanger, F., et al, Proc Natl Acad Sci (USA) (1977) 74:5463 as further described by Messing, et al, Nucleic

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Acids Res (1981) 9:309, or by the method of Maxam, et al, Methods in Enzymology (1980) 65:499.

Construction and Expression of DNA Sequences Encoding TNF Muteins

5 Retrieval of a clone encoding mature human TNF was described in PCT application US86/01921 (supra). This clone, pE4, is deposited at ATCC and has accession number 39894. The DNA sequence set forth in Figure 1 is contained in the insert to pE4.

10 As deduced from the cDNA sequence set forth in Figure 1, the mature TNF protein contains 157 amino acid residues, and has a molecular weight, without glycosylation, of approximately 17,354. The leader sequence apparently contains roughly 76 amino acids, 15 beginning with the first available Met start codon. There are 2 cysteine residues, at positions 69 and 101, leading to the possibility that the active structure contains a disulfide link.

20 Construction of bacterial expression vectors for the mature sequence and certain muteins thereof was described in the above-referenced PCT application. As intermediates to these vectors, the relevant sequences are transformed into M13 vectors as therein described.

25 Construction of M13-AW701 Substrate for Mutein-DNA Preparation

As described in the above-referenced application, the DNA fragment containing the upstream portion of the insert (containing the entire coding sequence but lacking some of the 3' untranslated region) 30 was excised from pE4 by digestion with PstI, isolated by agarose gel electrophoresis, recovered by electroelution, and ligated into the PstI site of bacteriophage M13mp18.

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The ligated phage were transduced into frozen competent E. coli K12 strain DG98 (ATCC #39768) and cultured by plating on media containing 5×10^{-4} M isopropyl thiogalactoside (IPTG) obtained from Sigma Chem. (St. Louis, MO) and 40 μ g/ml X-gal. Non α -complementing white plaques were picked onto fresh single strand phage DNA containing inserts of the expected (1.1 kb) size. The structure of the desired recombinant phage, designated clone 4.1, was confirmed using restriction analysis.

A chemically synthesized, purified, 33-mer oligodeoxyribonucleotide having the sequence:

5'-GAAGATGATCTGACCATGAGCTTGCCCTGGGCC-3'

was used to introduce a HindIII restriction enzyme site and an ATG-initiation codon before the GTC codon coding for the first amino acid (valine) of the mature TNF protein.

Ten picomoles of the oligonucleotide were hybridized to 2.6 μ g of ss clone 4.1 DNA in 15 μ l of a mixture containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.9, 20 mM MgCl₂ and 20 mM β -mercaptoethanol, by heating at 67°C for 5 min and 42°C for 25 min. The annealed mixtures were chilled on ice and then adjusted to a final volume of 25 μ l of a reaction mixture containing 0.5 mM of each dNTP, 17 mM Tris-HCl, pH 7.9, 17 mM MgCl₂, 83 mM NaCl, 17 mM β -mercaptoethanol, 5 units of DNA polymerase I Klenow fragment, incubated at 37°C for 1 hr. The reactions were terminated by heating to 80°C and the reaction mixtures used to transform competent DG98 cells, plated onto agar plates and incubated overnight to obtain phage plaques.

Plates containing mutagenized plaques, as well as plates containing unmutagenized phage plaques, were chilled to 4°C and phage plaques from each plate were transferred onto 2 nitrocellulose filter circles by

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layering a dry filter on the agar plate for 5 min for the first filter and 15 min for the second filter. The filters were then placed on thick filter papers soaked in 0.2 N NaOH, 1.5 M NaCl and 0.2% Triton X-100 for 5 min, and neutralized by layering onto filter papers soaked with 0.5 M Tris-HCl, pH 7.5, and 1.5 M NaCl for another 5 min. The filters were washed in a similar fashion twice on filters soaked in 2 x SSC, dried and then baked in a vacuum oven at 80°C for 2 hr. The 10 duplicate filters were prehybridized at 42°C for 4 hr with 10 ml per filter of DNA hybridization buffer (5 x SSC, pH 7.0, 4 x Denhardt's solution (polyvinyl pyrrolidone, Ficoll and bovine serum albumin, 1x = 0.02% of each), 0.1% SDS, 50 mM sodium phosphate buffer, pH 7.0 and 100 µg/ml of denatured salmon sperm DNA. 15 32 P-labeled probes were prepared by kinasing the primer with labeled ATP. The filters were hybridized to 5 x 10^6 cpm/ml of 32 P-labeled primer in 1-5 ml per filter of DNA hybridization buffer at 64°C for 8 hr.

20 The filters were washed once at room temperature for 10 min in 0.1% SDS, 20 mM sodium phosphate (buffer) and 6 x SSC; once at 37°C for 20 min in buffer and 2 x SSC; once at 50°C for 20 min in buffer and 2 x SSC; and finally at 60°C for 20 min in buffer and 1 x SSC. The filters were air dried and 25 autoradiographed at -70°C for 4 hr.

30 Since the oligonucleotide primer is designed to create a new HindIII restriction site in the mutagenized clones, RF-DNA from a number of the clones which hybridized with the primer were digested with this restriction enzyme. One of the mutagenized "clone 4.1" plaques which has a new HindIII restriction site (M13-AW701) was picked and inoculated into a culture of DG98, ssDNA was prepared from the culture supernatant and

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dsRF-DNA was prepared from the cell pellet. The correct sequence was confirmed by dideoxy sequencing.

5 The correctly synthesized strands were isolated and cleaved with PstI and HindIII (partial) or with HindIII alone for religation into the donor expression vector.

Construction of Expression Vectors for mTNF and Muteins

a. Construction of pAW701 and pAW702

10 For procaryotic expression under the control of the trp promoter, the coding sequence (along with some 3' untranslated nucleotides) was excised from dsM13-AW701 in two ways:

15 In the first method, the dsM13-AW701 was digested with PstI and then digested partially with HindIII to obtain the HindIII-PstI TNF coding sequence. (Partial HindIII digestion is required because there are several HindIII sites in M13-AW701.) The partial digestion of the DNA fragment can be accomplished by using one-tenth the amount of restriction enzyme required for complete digestion of the DNA. The mixture was incubated at the appropriate temperature for the enzyme, and aliquots of the digestion mixture were removed at 10 min intervals for up to 1 hr. The aliquots were then loaded onto a gel and the DNA fragments analyzed. The time point that provided the highest yield of the DNA fragment needed was chosen for a preparative digestion with the restriction enzyme and the appropriate fragment purified from the gel by electroelution.

20 30 The PstI/BamHI fragment containing the 3'-noncoding sequence of the TNF gene was purified from pE4 following digestion of the DNA with the enzymes PstI and BamHI.

Together, the HindIII/PstI and PstI/BamHI fragments comprise the coding sequence plus a 600 bp 3' untranslated portion of DNA. The two fragments were ligated into HindIII/BamHI-digested host vector pTRP3 as follows:

5 pTRP3 (ATCC 39946) contains the E. coli trp promoter and ribosome binding site. pTRP3 was digested with HindIII and BamHI, and the vector fragment purified on agarose gel. The isolated fragment was then ligated with the above HindIII/PstI and PstI/BamHI segments in a 10 three-way ligation, and the mixture used to transform E. coli MM294 to Amp^R, giving pAW701.

15 In a second method, dsM13-AW701 was digested with HindIII and the fragment containing the gene isolated on agarose gel. The isolated fragment was ligated with HindIII-cleaved, BAPped pTRP3, and transformed into E. coli MM294 to obtain pAW702.

15 b. Construction of pAW711 and Other PL Vectors

20 For prokaryotic expression under control of the PL promoter, alternative host vectors were employed for the TNF encoding DNA. Thus, pFC54.t (ATCC 39789) containing the PL promoter and *Bacillus* positive retroregulatory sequence was used as a host vector. This 25 vector was digested with HindIII and BamHI and the large plasmid fragment containing the control sequences purified on agarose gel. The HindIII/PstI and PstI/BamHI portions of the TNF gene, prepared as set forth above, were ligated in a three-way ligation with the HindIII/BamHI pFC54.t vector fragment, resulting in 30 plasmid pAW711. pPLOP (ATCC 39947) can also be used in an analogous manner to obtain pAW712.

Alternatively, the purified HindIII fragment from pE4 is ligated into HindIII-cleaved, BAPped pFC54.t or pPLOP to give pAW713 and pAW714, respectively.

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5 c. Construction of pAW711A and pAW736A

pAW711A is similar to pAW 711, but lacks most of the 3' noncoding DNA. To construct pAW711A, plasmid DNA of pAW711 was digested with restriction enzyme FokI, repaired with DNA polymerase I to create a blunt end, and then further digested with HindIII. An additional amount of pAW711 DNA was digested with BamHI and the cohesive end similarly repaired to form a blunt end and then further digested with HindIII. The HindIII/FokI (repair) fragment containing the TNF coding sequence and the large HindIII/BamHI (repair) fragment containing the plasmid vector were purified in a gel, eluted and ligated together with T4 DNA ligase under blunt-end ligation conditions. The resulting plasmid pAW711A has the BamHI site regenerated and the TNF coding sequence is within a HindIII/BamHI cassette fragment, with both HindIII and BamHI unique restriction sites. The 3'-noncoding region of the TNF cDNA has been deleted and the coding region brought closer to the retroregulator sequence.

10 Similarly, pAW736 (described below) was reconstructed to form pAW736A, with the coding sequence of the NV4-TNF mutein within a HindIII/BamHI cassette, having the 3'-noncoding region deleted.

15 d. Construction of pAW711C-HB

20 pAW711C-HB is a modification of pAW711A. To construct this vector, which contains tetracycline resistance marker and unique HindIII and BamHI sites, plasmid pAW711A was partially digested with EcoRI (pAW711A has two EcoRI restriction sites) and then further digested with EcoRV. The large EcoRI/EcoRV fragment containing the TNF coding sequence and most of the vector sequence was purified and eluted from a gel.

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The plasmid pBR322 was digested with *Ava*I and the cohesive end repaired by DNA polymerase I Klenow fragment to form a blunt-end and then further digested with *Eco*RI. The *Eco*RI/*Ava*I repaired fragment containing the coding sequence of the tetracycline gene was purified and ligated together with the purified large *Eco*RI/*Eco*RV fragment from pAW711A. The resulting plasmid pAW711C confers tetracycline resistance and contains two *Eco*RI sites, two *Hind*III sites, and two *Bam*HI sites.

The plasmid pAW711C was digested with *Eco*RI to delete the *Eco*RI fragment containing the *PL* promoter, the TNF coding sequence, and the *Bacillus* retroregulator sequence. The large *Eco*RI vector fragment was circularized by ligation, transformed into competent *E. coli*, and the tetracycline resistant colonies were screened for deletion of the TNF sequence. One such plasmid was identified as pAW710X. Plasmid pAW710X was mutagenized with bisulfite treatment and transformed into *E. coli*. Tetracycline resistant colonies were screened for the loss of the *Hind*III and *Bam*HI sites within the tetracycline resistance gene. One such plasmid was identified as pAW710-HB. The *Eco*RI fragment from pAW711A, containing the *PL* promoter, TNF coding sequence, and retroregulator sequence, was subcloned into the unique *Eco*RI site of pAW710-HB, resulting in the plasmid pAW711C-HB. This plasmid has unique *Hind*III and *Bam*HI restriction sites flanking the coding region for TNF and contains the tetracycline resistance marker.

Additional TNF Mutein Starting Materials

The M13-AW701 vector described above was used as the starting material for the synthesis of DNA encoding other TNF mutein starting materials.

5 a. Construction of M13-AW736 and pAW736

For example, to obtain the subclass wherein the first four N-terminal amino acids are deleted, a chemically synthesized, purified, 35-mer oligodeoxyribo nucleotide having the sequence:

5' -CACTCGGGGTTCGAGACATAAGCTTGCGCTGGGCC-3'

was used to loop out and thereby delete the 12 nucleotides encoding the four N-terminal amino acids downstream from the methionine initiation codon.

10 Ten picomoles of the oligonucleotide were hybridized to 2.6 µg of ss clone M13-AW701 DNA in 15 µl of a mixture containing 100 mM NaCl, 20 mM Tris-HCl, pH 7.9, 20 mM MgCl₂ and 20 mM β -mercaptoethanol, by heating at 67°C for 5 min and 42°C for 25 min. The annealed mixtures were chilled on ice and then adjusted to a final volume of 25 µl of a reaction mixture containing 0.5 mM of each dNTP, 17 mM Tris-HCl, pH 7.9, 17 mM MgCl₂, 83 mM NaCl, 17 mM β -mercaptoethanol, 5 units of DNA polymerase I Klenow fragment, incubated at 37°C for 15 20 1 hr. The reactions were terminated by heating to 80°C and the reaction mixtures used to transform competent DG98 cells, plated onto agar plates and incubated overnight to obtain phage plaques.

25 Plates containing mutagenized clone M13-AW701 plaques, as well as 2 plates containing unmutagenized clone M13-AW701 phage plaques, were chilled to 4°C, and phage plaques from each plate were transferred onto 2 nitrocellulose filter circles by layering a dry filter on the agar plate for 5 min for the first filter and 15 min for the second filter. The filters were then placed on thick filter papers soaked in 0.2 N NaOH, 1.5 M NaCl and 0.2% Triton X-100 for 5 min, and neutralized by layering onto filter papers soaked with 0.5 M Tris-HCl, pH 7.5, and 1.5 M NaCl for another 5 min. The filters 30 35 were washed in a similar fashion twice on filters soaked

in 2 x SSC, dried and then baked in a vacuum oven at 80°C for 2 hr. The duplicate filters were prehybridized at 42°C for 4 hr with 10 ml per filter of DNA hybridization buffer (5 x SSC, pH 7.0, 4 x Denhardts solution (polyvinyl pyrrolidone, Ficoll and bovine serum albumin, 1x = 0.02% of each), 0.1% SDS, 50 mM sodium phosphate buffer, pH 7.0 and 100 µg/ml of denatured salmon sperm DNA. 32 P-labeled probes were prepared by kinasing the primer with labeled ATP. The filters were hybridized to 5 x 10^6 cpm/ml of 32 P-labeled primer in 1-5 ml per filter of DNA hybridization buffer at 64°C for 8 hr.

The filters were washed once at room temperature for 10 min in 0.1% SDS, 20 mM sodium phosphate (buffer) and 6 x SSC; once at 37°C for 20 min in buffer and 2 x SSC; once at 50°C for 20 min in buffer and 2 x SSC; and finally at 60°C for 20 min in buffer and 1 x SSC. The filters were air dried and autoradiographed at -70°C for 4 hr. Several positive plaques were obtained, and one, designated M13-AW736, was used to construct expression vectors and as a starting material for constructing DNA encoding certain muteins of the invention.

To construct the expression vectors, RF-DNAs from positive clones were digested with HindIII, and the fragment containing the mutagenized TNF coding sequence was isolated by gel electrophoresis. The recovered sequence was ligated into HindIII cleaved, BAPped pAW711 to obtain pAW736. The presence of the 12 nucleotide deletion was confirmed by restriction analysis with HindIII and PvuII; pAW736 contains a 134 bp HindIII/PvuII fragment as compared to the 146 bp HindIII/PvuII fragment produced by pAW711. pAW736 was deposited with ATCC on 10 April 1985 and has accession no. 53092.

5 b. Other Starting Materials

In a manner precisely similar to that set forth above, M13 vectors and expression vectors were prepared for TNF deletion muteins lacking the first 3-11 amino terminal residues as compared with the sequence shown in Figure 1. (pAW711 was used as the host vector in all cases.) Figure 2 shows the designations of the resulting vectors and the oligomers used in the site-specific mutagenesis reactions used to create the deletions. This figure also shows the vector designations and oligomers used to construct CV156⁻, CV150⁻, and CV140⁻-TNF, as well as the des-ser₃des-ser₄ and val₁₅ser₁₆ muteins.

10 In addition, the HindIII/BamHI fragments from pAW711A and pAW736A were cloned into M13mp8 to obtain M13-AW711A and M13-AW736A, respectively.

15 Preparation of DNA Encoding Arginine-Depleted Muteins

20 In a manner precisely analogous to that described above, using M13-AW701, M13-AW736, M13-711A, or the other corresponding modified M13 vectors as starting materials, the DNAs encoding the arginine-depleted muteins of the invention are prepared and the mutated DNAs transferred into pAW711, pAW711A, or pAW711C-HB as host vector, as was described for the preparation of pAW736. Figure 3 shows the designations of the resulting expression vectors and the DNA sequences of the oligomers used to perform the mutations for representative muteins of the invention. All of the vectors shown in Figure 3 were constructed, expressed, and demonstrated to produce protein having TNF activity; all can be constructed as described herein from deposited starting materials.

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5 Specifically, pAW748, pAW749, and pAW750, which are effective in expressing leu₁glu₂-TNF, leu₁-TNF, and glu₂-TNF, respectively, were prepared using M13-AW701 as starting material, and the resultant mutated segments ligated into pFC54.t as described for the preparation of pAW711 above.

10 The plasmids pAW756A (lys₂-TNF) and pAW765A (glu₂-TNF) were analogously constructed, but using M13-AW711A as starting material and switching the HindIII/BamHI fragment with pAW711A.

15 The plasmids pAW787C-HB, pAW788C-HB through pAW791C-HB, pMN796C-HB and pMN801C-HB were analogously constructed, but using M13-AW711A as starting material and switching the HindIII/BamHI fragment with pAW711C-HB.

20 The DNA sequence encoding a cysteine-69 and/or cysteine-101-substituted TNF mutein may be used to modify N-terminal deleted muteins to obtain the corresponding "double" mutein forms. The expression vectors for these muteins are constructed by using appropriate restriction enzymes to fragment switch portions of the DNA encoding regions containing these modifications into the vectors prepared above. The regions containing the ser₆₉, ser₁₀₁, and ser₆₉ser₁₀₁TNF 25 forms are obtained from plasmids pAW731, pAW732, or pAW735, respectively. The plasmid pAW731, in particular, has been deposited at ATCC on 25 January 1985 with accession no. 53007. The expression vectors thus obtained are transformed into E. coli and the cells cultured and induced as above to produce the desired 30 proteins. The resulting TNF muteins are comparably active to mTNF.

Production of Arginine-Deleted Muteins

5 The expression vectors described containing the coding sequences for the various TNF muteins, including those of the invention shown in Figure 3, under control of the P_L promoter were transfected into E. coli MC1000-39531, a lambda lysogen, and the cells were induced at high temperature. After several hours of culturing under induction conditions, the cells were 10 sonicated and sonicates verified to contain TNF by the L-929 cytotoxicity assay.

15 The TNF produced can then be purified as follows: E. coli DG95 (a lambda lysogen similar to MC1000-39531) transformed with the appropriate expression vector are grown at 37°C in standard growth medium to an OD₆₀₀ of about 0.5 and then induced by increasing the temperature to 42°C. After 2 hr, the cells are sonicated and the sonicate is verified to 20 contain TNF activity using the L-929 cytotoxicity assay (supra). The sonicate is then applied to a DEAE Sepharose column (Pharmacia) and washed with buffer (10 mM Tris, pH 8.2, 1 mM NaCl). Stepwise elution with 0.02 M, 0.04 M, 0.1 M, and 0.8 M NaCl in 10 mM Tris, pH 8.2 yields fractions containing TNF activity.

25 Most of the TNF activity elutes at 0.04 M NaCl. These fractions are concentrated by ultrafiltration, and then further purified by HPLC using a phenyl TSK-5PW column (LKB). The TNF protein is bound to the column in the presence of 1.8 M ammonium sulfate in 0.1 M sodium phosphate, pH 7.0, and is eluted at 30 approx. 0.4 ammonium sulfate when the column was developed by reducing the ammonium sulfate concentration to zero. The fractions containing TNF are concentrated by ultrafiltration and applied to a GH25 sizing column (Amicon) to obtain pure TNF.

When mTNF was encoded, i.e., when pAW711 was used, isoelectric focusing showed the TNF to consist of several species of differing pI values in the range of 5.8-6.5. All major species were shown to be the expected mature TNF (mTNF) but a contaminant mutein form NV4TNF was also present. The results of isoelectric focusing gel show multiple modifications of TNF to be present.

On the other hand, isoelectric focusing of various muteins showed more homogeneous forms. Figure 4 shows the results of isoelectric focusing performed on purified lysates from DG95 E. coli transformed with pAW711 (mTNF); pAW740 (NV8-TNF); pAW748 (leu₁glu₂-TNF); pAW749 (leu₁-TNF); pAW750 (glu₂-TNF); and pAW756 (lys₂-TNF). It is clear from the results in Figure 4 that mTNF, leu₁-TNF, and lys₂-TNF, all of which contain basic amino acids in position 2, give heterogeneous proteins with multiple bands in the IEF. The number of bands is greatly reduced for the NV8-TNF (in which arginine at both the 2 and 6 positions has automatically been deleted) and for glu₂-TNF and leu₁glu₂-TNF, also free of arginine at position 2. (All of these preparations appear homogeneous when subjected to SDS-PAGE, as shown in Figure 5.)

Similar results are obtained in isoelectric focusing of NV4-TNF, in which the arginine at position 2 has automatically been deleted, but not that at position 6, in comparison with muteins containing substitutions at position 6 for the arginine. Thus, as shown in Figure 6, IEF of pAW711 and pAW736 which encode mTNF and NV4-TNF, respectively, results in multiple bands, whereas only single bands are obtained for pAW788C-HB (NV4glu₆-TNF); pAW789C-HB (NV4pro₆-TNF); pAW790C-HB (NV4asp₆-TNF) and pAW791C-HB (NV4ser₆-TNF). The preparations giving the highest level of homogeneity,

indeed showing only one band, are those for NV4glu6-TNF and NV4asp6-TNF wherein the arginine at position 6 has been substituted by an acidic amino acid.

5 Thus, representative muteins of the invention show greater stability to side chain alteration, as evidenced by their IEF behavior.

The muteins of the invention thus purified can be used pharmacologically as described hereinabove.

10 An additional advantage to certain muteins of the invention relates to processing of the N-terminal methionine. When the plasmids encoding NV4asp6-TNF and NV4ser6-TNF are expressed in E. coli, the N-terminal methionine is completely removed (pAW790C-HB and pAW791C-HB). When those encoding NV4glu6-TNF (pAW788C-HB) or NV4pro6-TNF (pAW789C-HB) are expressed, 30% and 15 65% respectively of the proteins produced retain the N-terminal met.

20 The following plasmids and hosts were deposited with Cetus Master Culture Collection (CMCC) of the assignee and the American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD, USA (ATCC). These 25 deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture for 30 years from date of deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Applicants 30 and ATCC which assures unrestricted availability upon issuance of the pertinent US patent. Availability of the deposited strains is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government 35 in accordance with its patent laws.

Provision of deposits is for the convenience of practitioners and does not constitute an admission that the written word as presented in the specification is non-enabling. It is believed that the deposited materials are sufficiently described in the specification to permit their construction by one of ordinary skill in the art or are known in the art. Furthermore, a number of plasmids whose construction is described in the specification and shown in Figure 3 are not deposited with ATCC. However, these plasmids are also easily constructed using the written description herein and have been made, expressed, and confirmed to produce active TNF protein.

	<u>Plasmid</u>	<u>CMCC No</u>	<u>ATCC No</u>	<u>Deposit Date</u>
15	pPLOP	2118	39947	18 December 1984
	pTRP3	1731	39946	18 December 1984
	<u>E. coli</u> DG98	1965	39768	13 July
	pFC54.t	2103	39789	7 August 1984
	pE4/ <u>E. coli</u> MM294	2318	39894	15 October 1984
20	pAW711/ <u>E. coli</u> DG95	2162	39918	8 November 1984
	pAW731	2219	53007	25 January 1985
	pAW736/ <u>E. coli</u> DG95	2317	53092	10 April 1985
	pAW748	2669	67218	25 September 1986
	pAW750	2671	67219	25 September 1986
25	pAW765A	2680	67316	10 February 1987
	pAW788C-HB	2908	67317	10 February 1987
	pAW790C-HB	2910	()	12 January 1988
	pAW791C-HB	2911	()	12 January 1988

-40-

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A human TNF protein which is modified from the amino acid sequence shown in Figure 1, including the naturally occurring allelic variants thereof, wherein said modifications are selected from the group consisting of:

(1) deletion of or substitution for arginine at position 6;

(2) deletion of or substitution for arginine at position 6 in combination with an N-terminal deletion of 1-5 amino acids;

(3) deletion of or substitution for arginine at position 2;

(4) deletion of or substitution for arginine at position 2 in combination with deletion of amino acid 1;

(5) deletion of or substitution for arginine at both positions 2 and 6;

(6) deletion of or substitution for arginine at both positions 2 and 6 in combination with deletion of amino acid 1;

(7) deletion of or substitution for arginine at position 31, 32, or both;

(8) deletion of or substitution for arginine at position 31, 32, or both in combination with deletion of 1-10 amino acids from the N terminus;

(9) deletion or substitution for arginine at position 31, 32, or both in combination with one of the modifications set forth in (1)-(8) above;

(10) substitution of valine at position 1 with a neutral amino acid; and

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(11) substitution for valine at position 1 with a neutral amino acid in combination with one of (1), (3), (5) or (7),

wherein the amino acid substituted for arginine is a neutral or acidic amino acid.

2. The modified human TNF of claim 1 which further includes substitution of the cysteine residues at position 69, 101, or both.

3. The protein of claim 1 wherein the neutral or acidic amino acid substituted for arginine is glu, gln, asp, asn, ser, pro, or gly.

4. The protein of claim 3 wherein the neutral or acidic amino acid substituted at position 2 and/or 6 is glu or asp and that substituted at position 31 and/or 32 is asn or gln.

5. The protein of claim 1 wherein the neutral or acidic amino acid substituted at position 1 is leu or ile.

6. The protein of claim 1 which is selected from the group consisting of pro₂-TNF, N₇4 glu₆-TNF, N₇4 pro₆-TNF, N₇4 asp₆-TNF, N₇4 ser₆-TNF, leu₁glu₂-TNF, leu₁TNF, glu₂-TNF, gln₃1-TNF, gln₃2-TNF, gln₃1gln₃2-TNF, gly₆-TNF, ser₂ser₆-TNF, gly₂gly₆-TNF, glu₂glu₆-TNF, and asp₂asp₆-TNF.

7. The protein of claim 6 which is N₇4asp₆-TNF.

8. The protein of claim 6 which is N₇4ser₆-TNF.

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9. A recombinant DNA sequence which encodes the protein of claim 1.

10. A recombinant DNA sequence containing control sequences for expression in a suitable host operably linked to the DNA sequence of claim 9.

11. A recombinant host cell transformed with the DNA sequence of claim 10.

12. The cell of claim 11, which is a bacterium.

13. A transformation vector capable of conferring on a transformant host the ability to express the TNF protein of claim 1.

14. The transformation vector of claim 13 which comprises plasmid pAW790C-HB.

15. The transformation vector of claim 13 which comprises plasmid pAW791C-HB.

16. A recombinant host transformed with the vector of claim 13.

17. A method of producing the protein of claim 1 which comprises culturing cells transformed with an expression system comprising recombinant DNA encoding said protein, culturing said cells, inducing the production of the protein, and recovering said protein from the cells.

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18. A pharmaceutical composition comprising the protein of claim 1 in combination with a pharmaceutically acceptable carrier.

19. A method to treat tumor burden in mammals which comprises administering to a subject in need of such treatment a pharmacologically effective amount of the protein of claim 1.

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FIG. 1 - 1

1 CACACCCGTGACAAAGCTGCCAGGGCAGGTCTCTCTCACATACTGACCCCACGGGCTCCA

61 CCCTCTCTCCCTGGAAAGGACACCATGAGCACACTGAAAGCATGATCCGGACGTGGAGCT
AlaGluGluAlaLeuProLysSerThrGluSerMetIleArgAspValGluLeu

121 GCCCGAGGGGCTCCCAAGAACAGGGGGCCACAGGGGCTCCAGGGCTCTGGGGTGTGTTGTT
AlaGluGluAlaLeuProLysSerThrGlyGlyProGlnGlySerArgArgCysLeuPhe

181 CCTCAGCCTCTCTCCTGATCGTGGAGGGGCCACCACGGCTCTCTGCCTGCTGCA
LeuSerLeuPheSerPheLeuIlevalAlaGlyAlaThrThrLysSerArgArgCysLeuLeuIle

241 CTTGGAGTGTGACGGCCCCCAGAGGGAAAGAGTCCCCAGGGACCTCTCTAAATCAGCCC
PheGlyValIleGlyProGlnArgGluSerProArgAspLeuSerLeuIleSerPro

01 TCTGGCCAGGCAGTCAGATCATCTCTCGAACCCGAGTGACAAGGCCATGTTAGCCCATG
LeuAlaGlnAlaValArgSerSerSerArgThrProSerAspLysProValAlaHisVal

6 1 TGTAGCAAACCCCTCAAGCTGAGGGCAGCTCCAGTGGCTGAAACCGCCGGCCAAATGCCCT
ValAlaAsnProGlnAlaGluGlyGlnLeuGlnTrpLeuAsnArgArgAlaAsnAlaLeu

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421 CCTGGCCAATGGCGTGGAGCTGACAGATAACCAGCTGGTGGCCATCAGAGGGCCCTGTA
LeuAlaAsnGlyValGluLeuArgAspSerGlnLeuValValProSerGluGlyLeutyr

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601 601

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LeuIleTyrSerGlnValLeuPheLysGlyGlnGlyCysProSerThrHisValLeuLeu

LeuIleTyrSerGlnValLeuPheLysGlyGlnGlyCysProSerThrHisValLeuLeu

ThrIleSerSerArgIleAlaValSerTyrGlnThrLysValAsnLeuSerAla^{1a}

IleLysSerProCysGlnArgGluThrProGluGlyAlaGluAlaLysProTrpTyrGlu

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FIG. 1 -2

661 GCCCATCTATCTGGGGGCTCCAGCTGGAGAAGGGTACCGACTCAGGGCTGAGAT
 Pro Ile Tyr Leu Gly Gly Val Phe Glu Ile Asp Arg Leu Ser Ala Glu Ile
 101 106 111 116
 121 126 131 136
 721 CAATCGGGCCGACTATCTCGACTTTGCCCAGGTCTGCTACTTTGGGATCATTC
 Asn Arg Pro Asp Tyrolyle Asp Phe Ala Glu Ile Ser Gly Glu Val Ile Ala
 141 146 151 156
 781 CCTGTGAGGGAGCAACATCCAAACCTTCCAAACGGCCTCCCCATGCCCAATCCCTTAT
 Leu . . .

841 TACCCCCCTCCCTCAGACACCCCTAACCTCTGGCTCAAAAGAGAAATTGGGGGCTTAG
 901 GGTCGGAAACCCAAAGCTTAGAACTTTAACGCAACAAAGACCACCACTTCGAAACCTGGGATTC
 961 AGGAATGTGTGGCCTGCACAGTGAAGTGTGGCAACCACTAACAAACTGGGCCCT
 1021 CCAGAAACTCACTGGGCCCTACAGCTTTGATCCCTGACATCTGGAAATCTGGAGACAGGGAA
 1081 GCCTTGTGTCTGGCCAGAAATGCTGCAGGACTTGAGAAGAACCTCACCTAGAAATTGACAC
 1141 AAGTGGACCTTAGGCCTTCCTCCAGATGTTCCAGACTTCCTTGAGACACGGAGCC
 1201 CAGCCCTCCCCATGGAGCCAGCTCCCTCTATTATGTAATTGTTGACTTGTGATTATTATTAT
 1261 TTATTATTATTATTTACAGATGAAATGTTGTTGAGACACGGGGTATCCCTG
 1321 GGGGACCCAAATGTAGGAGCTGCCCTGGCTCAGACATGTTTCCGTGAAAACGGAGGCTGA
 1381 ACAATAGGCTGTTCCCATGCCCCCTGGCCTCTGTGCTCTTGATTATGTTTT
 1441 AAAATATTATCTGATTAAAGTGTCTAAACAAATGCTGATTGGTGAACCAACTGTCACTCAT
 1501 TGCTGAGGCCCTCTGCTCCCCAGGGACTTGTCCTGTAATCGGGCTACTATCAGTGGCGA

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FIG. 2

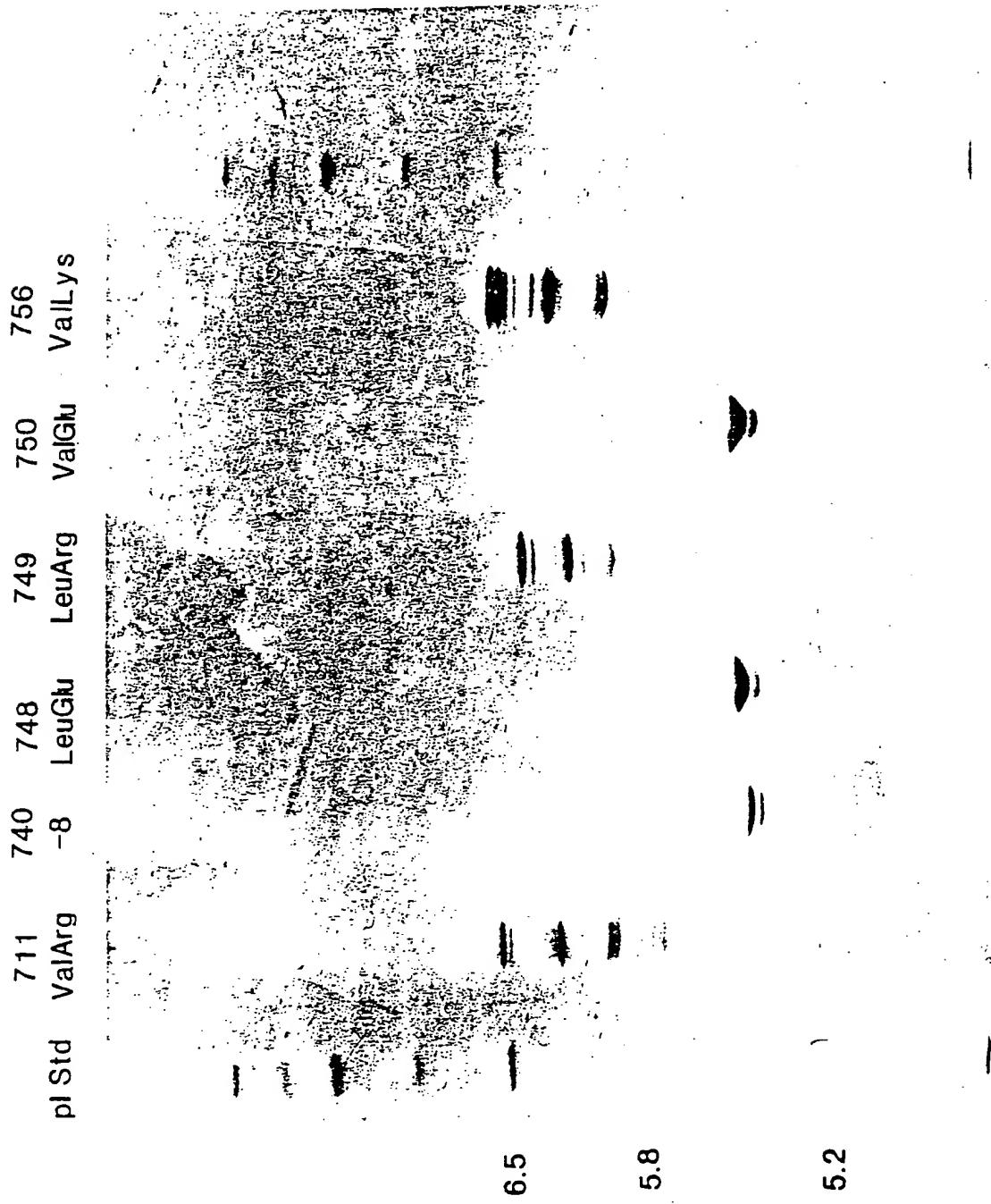
<u>TNF</u>	<u>Mutant</u>	<u>Plasmid</u>	<u>Oligomer</u>
mTNF		pAW711	-----
74		pAW736	CACTCGGGGGTTCGAGACATAAGCTTTGCCTGGCC
75		pAW738	GCTTGTCACTCGGGGTCATTAAGCTTTGCC
76		pAW739	GCTTGTCACTCGGGGTCATTAAGCTTTGCC
77		pAW737	CAGGGCTTGTCACTCGGGCATTAAGCTTTGCCTGGCC
78		pAW740	CTACAGGGCTTGTCACTCATTAAGCTTTGCCTGGCC
79		pAW741	GGGCTACAGGGCTTGTICATTAAGCTTTGCCTGGCC
80		pAW742	CATGGGCTACAGGGCTCATTAAGCTTTGCCTGGCC
81		pAW743	CAACATGGGCTACAGGGCATTAAGCTTTGCCTGGCC
82		pAW744	GAGGGTTTGTCAACCATTAAGCTTTGCCTGGCC
83		pAW745	GATGTTCGTCTCCTCAGGCAATGATCCAAAG
84		pAW746	GTATGTTGTCTCCTCAGACTGCCAGACTCGGC
85		pAW747	GTATGTTGTCTCCTCAGTCGGCCGATTGATCTC
86		pAW733	GGGTTCGAGAACGGACCATAACG
87		pAW734	GTTCGCTACAGAACGGCTAC
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FIG. 3

<u>TNF Encoded</u>	<u>Plasmid</u>	<u>Oligomer Primer</u>
mTNF	PAW711	GAAGATGATCTGACCATAAGCTTGCCTGGGCC
pro ₂ -TNF	PMN787C-HB	CGAGAAGATGGGACCATAAAGCTTTGC
NV4 glu ₆ -TNF	PAW788C-HB	GTCACTCGGGGTTCAAGACATAAGCTTGC
NV4 pro ₆ -TNF	PAW789C-HB	GTCACTCGGGGTTGGAGACATAAGCTTGC
NV4 asp ₆ -TNF	PAW790C-HB	GTCACTCGGGGTTAGACATAAGCTTGC
NV4 ser ₆ -TNF	PAW791C-HB	GTCACTCGGGGTTGAAGACATAAGCTTGC
leu ₁ glu ₂ -TNF	PAW748	GAAGATGATTCCAGCATAAGCTTTGC
leu ₁ -TNF	PAW749	GATGATCTCAGCATAAGCTTTGC
glu ₂ -TNF	PAW750	GAAGATGATTGACCATAAGC
lys ₂ -TNF	PAW756A	CGAGAAGATGACTTGACCATAAAGC
glu ₃₁ -TNF	PAW765A	GCATTGGGGCTGGTTAGCC
NV4 his ₆ -TNF	PMN796C-HB	GTCACTCGGGGTATGAGACATAAGCTTGC
NV4 gly ₆ -TNF	PMN801C-HB	GTCACTCGGGGTTCCAGACATAAGCTTGC

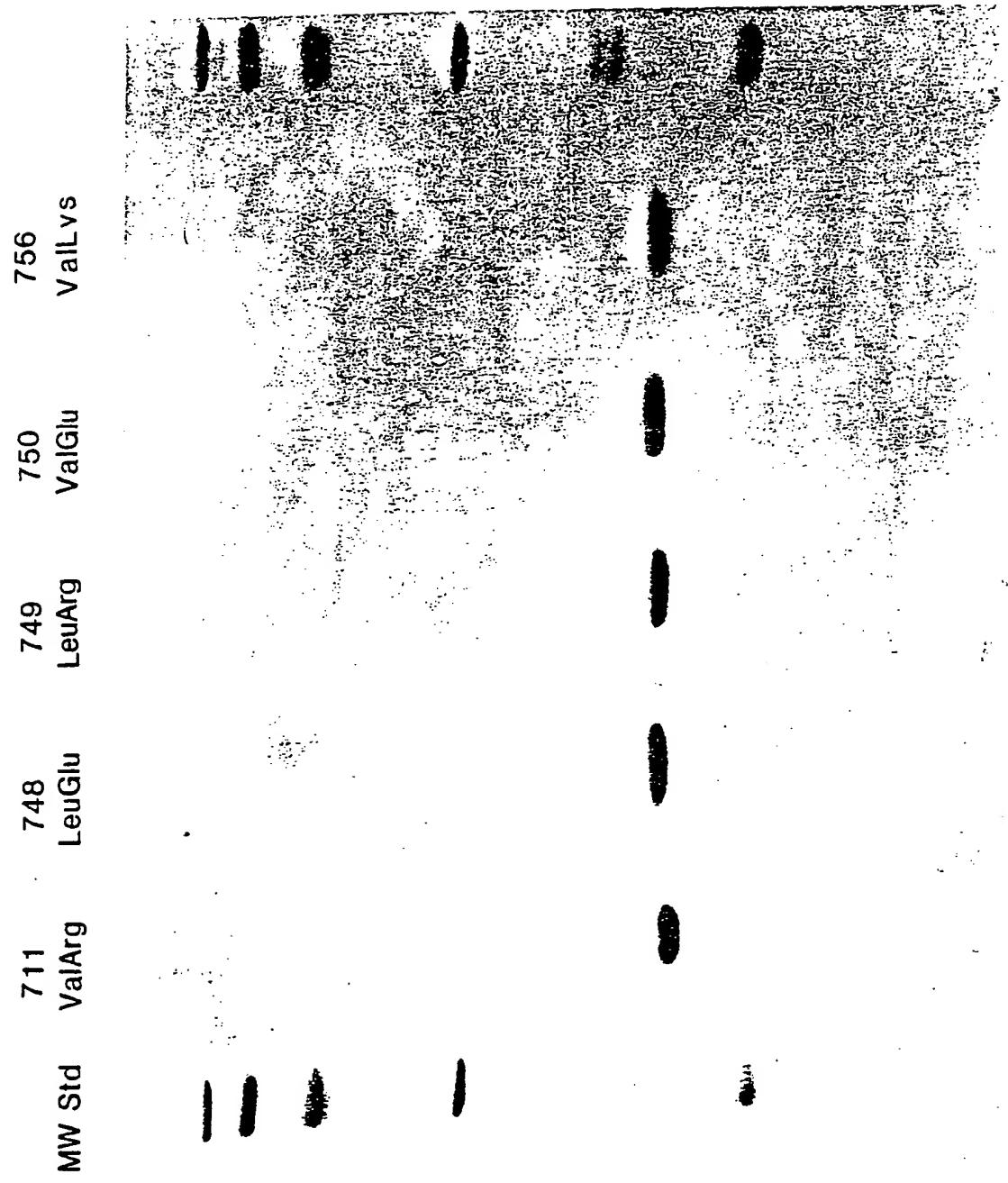
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FIG. 4 IEF of TNF MUTEINS



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FIG. 5 SDS/PAGE of TNF MUTEINS

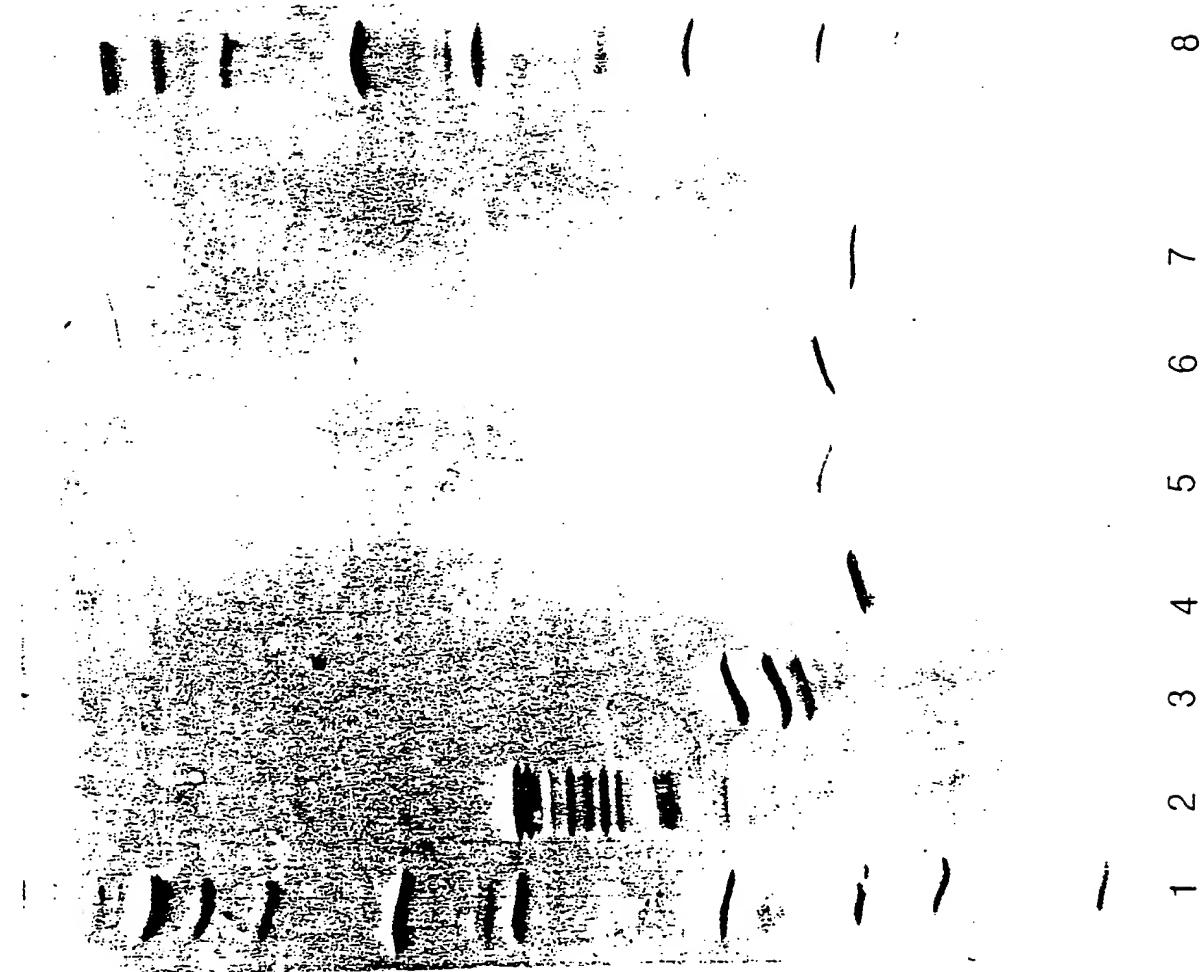


SUBSTITUTE SHEET

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LANE 1: MARKERS
LANE 2: PAM 711
LANE 3: PAM 736
LANE 4: PAM 788C-HB
LANE 5: PAM 789C-HB
LANE 6: PAM 791C-HB
LANE 7: PAM 790C-HB
LANE 8: MARKERS

FIG. 6 IEF of TNF MUTEINS





INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁴ : C12N 15/00, C12P 21/02 A61K 37/02		A3	(11) International Publication Number: WO 88/06625 (43) International Publication Date: 7 September 1988 (07.09.88)
(21) International Application Number: PCT/US88/00183		(74) Agent: HALLUIN, Albert, P.; Cetus Corporation, 1400 Fifty-Third Street, Emeryville, CA 94608 (US)	
(22) International Filing Date: 25 January 1988 (25.01.88)		(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent).	
(31) Priority Application Number: 019,221		(32) Priority Date: 26 February 1987 (26.02.87)	
(33) Priority Country: US		(71) Applicant: CETUS CORPORATION [US/US]; 1400 Fifty-Third Street, Emeryville, CA 94608 (US).	
(72) Inventors: MARK, David, F. ; 217 Stanbridge Court, Danville, CA 94526 (US). LIN, Leo, S. ; 1317 Chesterton Way, Walnut Creek, CA 94596 (US). THOMSON, James, W. ; 811 Talbot Avenue, Albany, CA 94706 (US). YAMAMOTO, Ralph ; 423 Pine Park Court, Martinez, CA 94553 (US).		<p>Published <i>With international search report Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p>(88) Date of publication of the international search report: 20 October 1988 (20.10.88)</p>	

(54) Title: ARGinine-DEPLETED HUMAN TUMOR NECROSIS FACTOR

<u>TNF Mutein</u>	<u>Plasmid</u>	<u>Oligomer</u>
mTNF	pAW711	-----
¶4	pAW736	CACTCGGGGTTCGAGACATAAGCTTGCTGGGCC
¶5	pAW738	GCTTGTCACTCGGGGTTCGCATAAGCTTGCC
¶6	pAW739	GCTTGTCACTCGGGGTCATAAGCTTGCC
¶7	pAW737	CAGGCTTGTCACTCGGCATAAGCTTGCTGGGCC
¶8	pAW740	CTACAGGCTTGTCACTCATAGCTTGCTGGGCC
¶9	pAW741	GGGCTACAGGCTTGTCCATAAGCTTGCTGGGCC
¶10	pAW742	CATGGGCTACAGGCTTCATAAGCTTGCTGGGCC
¶11	pAW743	CAACATGGGCTACAGGCTATAAGCTTGCTGGGCC
¶15	pAW744	GAGGGTTTGTACAACCATAGCTTGCTGGGCC
¶156-	pAW745	GATGTTCGTCCCTCCTCAGGCAATGATCCAAAG
¶150-	pAW746	GTATGTTCGTCCCTCCTCAGACCTGCCAGACTGGC
¶140-	pAW747	GTATGTTCGTCCCTCCTCAGTCGGGCCATTGATCTC
desSer ₃ desSer ₄	pAW733	GGGTTGAGAACGGACCATAAGC
Val ₁₅ Ser ₁₆	pAW734	GTTTGCATACAGAAACGGCTAC

(57) Abstract

Muteins of tumor necrosis factor (TNF) which are arginine depleted are biologically active and have superior handling properties. Particularly preferred are deletions of, and substitutions by, neutral or acidic amino acids for the arginine residues at positions (2 and 6) of mature TNF. These muteins have higher homogeneity when subjected to isoelectric focusing. Deletion and substitution of arginine at positions (31 and 32) results in TNF muteins which are stable to the action of proteases.

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CM Cameroon	LU Luxembourg	TD Chad
DE Germany, Federal Republic of	MC Monaco	TG Togo
DK Denmark	MG Madagascar	US United States of America
FI Finland		

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 88/00183

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁴

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁴: C 12 N 15/00; C 12 P 21/02; A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
IPC ⁴	C 12 N; C 12 P
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸	

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	EP, A, 0168214 (GENENTECH INC.) 15 January 1986 see page 17, line 7-11; page 22, line 14 - page 24, line 35; page 63, line 9; page 65, line 8; page 65, line 11	1, 2, ..., 5, 16-18
Y	cited in the application ---	2
X	EP, A, 0155549 (DAINIPPON PHARMACEUTICAL CO.) 25 September 1985 see page 16, line 29 - page 19; line 13; page 21, line 6 - line 19 cited in the application ---	1, 9, 10-13, 16-18
X, P	EP, A, 0247906 (MIZUNO AND SOMA) 2 December 1987 see page 6, line 27 - page 7, line 28; page 8, line 28 - page 9, line 10; page 17, line 36; claim 15 ---	1, 3, 4, 5, 16-18
		. / .

* Special categories of cited documents: ¹⁰

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"4" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

30th August 1988

International Searching Authority

EUROPEAN PATENT OFFICE

Date of Mailing of this International Search Report

21 SEP 1988

Signature of Authorized Officer

P.C.G. VAN DER PUTTEN

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	WO, A, 86/02381 (CETUS CORPORATION) 24 April 1986 see page 42, line 14 - page 45, line 10 claims 5,12 cited in the application	1,9,10-13, 16-18
Y	WO, A, 86/04606 (CETUS CORPORATION) 14 August 1986 see claims 6-8 cited in the application	2

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers 19..... because they relate to subject matter not required to be searched by this Authority, namely:

..... PCT Rule 39.1(iv):

Methods for treatment of the human or animal body by means of surgery or therapy, as well as diagnostic methods.

2. Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this International application as follows:

Part 1 - Claims 1-2(partially) 3,4,5-6(partially), 7,8, 9-13 (partially), 14,15, 16-18 (partially)

Part 2 - Claims 1-2 (partially), 5-6 (partially) 9-13 (partially) 16-18 (partially)
See Form PCT/ISA 206 dated 20th June 1988.

1. As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application.

2. As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

The additional search fees were accompanied by applicant's protest.

No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT
 ON INTERNATIONAL PATENT APPLICATION NO. US 8800183
 SA 20967

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 12/09/88. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0168214	15-01-86	AU-A-	4465285	09-01-86
		JP-A-	61040221	26-02-86
		US-A-	4650674	17-03-87
EP-A- 0155549	25-09-85	JP-A-	60185799	21-09-85
		AU-A-	3944885	12-09-85
		JP-A-	60232097	18-11-85
		JP-A-	61050923	13-03-86
EP-A- 0247906	02-12-87	None		
WO-A- 8602381	24-04-86	AU-A-	5010685	02-05-86
		EP-A-	0200748	12-11-86
		JP-T-	62500631	19-03-87
		US-A-	4677064	30-06-87
		US-A-	4677063	30-06-87
WO-A- 8604606	14-08-86	AU-A-	5518086	26-08-86
		EP-A-	0213175	11-03-87
		JP-T-	62501608	02-07-87

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